Variable Airway Responsiveness to Inhaled Lipopolysaccharide

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Individuals exposed to inhaled endotoxin (lipopolysaccharide [LPS]) can develop airway symptomatology and exacerbations of asthma. Moreover, among those occupationally exposed to organic dusts, the progression of airflow obstruction is related to the endotoxin concentration in the bioaerosol. Not everyone exposed to high concentrations of LPS develops these problems. To determine whether individuals express a differential response to inhaled LPS, we challenged 72 healthy volunteers with increasing doses of LPS. Airflow was assessed after each dose and the protocol was terminated for decline in $FEV_1 \geqslant 20\%$. Marked differences in the response to inhaled LPS were observed: eight "sensitive" subjects had at least 20% decline in their FEV_1 after inhaling 6.5 μg or less of LPS, whereas 11 "hyporesponsive" subjects maintained an $FEV_1 \geqslant 90\%$ of their baseline even after inhaling 41.5 μg of LPS. Serial testing demonstrated that the response to inhaled LPS is reproducible. Sensitive subjects were more commonly female and hyporesponsive subjects were more often male ($p = 0.016$). Peripheral blood monocytes from hyporesponsive subjects, compared with sensitive subjects, released less interleukin (IL)-6 and IL-8. These findings demonstrate that an LPS phenotype can be reproducibly elicited in humans, which creates an opportunity to identify genes involved in this response to inhaled LPS. Kline JN, Cowden JD, Hunninghake GW, Schutte BC, Watt JL, Wohlford-Lenane CL, Powers LS, Jones MP, Schwartz DA. Variable airway responsiveness to inhaled lipopolysaccharide.

AM J RESPIR CRIT CARE MED 1999;160:297–303.

Asthma, a disorder characterized by inflammation of the airways, is an increasing cause of morbidity and mortality in the United States, particularly among children (1). The cause for its rising severity is unknown, although factors as disparate as poor access to medical care and allergic responses to cockroach antigens (2) have been cited. Factors that induce or perpetuate the inflammatory response have an adverse effect on asthma outcomes. Although environmental allergens have been associated with increased asthma severity and frequency of exacerbations, the role of other inhaled agents is less clearly defined. Endotoxin, a cell wall component of gram-negative bacteria that is a lipopolysaccharide (LPS), is ubiquitous in the environment, and is often present in high concentrations in organic dusts (3) as well as in air pollution. A potent inflammatory agent, endotoxin may play an important role in the initiation or promotion of the airway inflammation in asthma.

Several lines of evidence indicate that endotoxin is an important component of the bioaerosol that contributes to airway inflammation and airflow obstruction. First, the concentration of inhaled endotoxin in the bioaerosol is strongly associated with the development of acute decrements in airflow among cotton workers (4) and swine confinement workers (5). The concentration of endotoxin in the bioaerosol is the most important occupational exposure associated with the development (6) and progression (7) of airway disease in agricultural workers. Second, physiologically, inhaled endotoxin (8, 9) and grain dust (10) can cause airflow obstruction in naïve or previously unexposed subjects. Naïve, healthy study subjects challenged with dust from animal confinement buildings develop airflow obstruction and an increase in the serum concentration of neutrophils and interleukin (IL)-6, all of which are most strongly associated with the concentration of endotoxin (not dust) in the bioaerosol (11). Third, our previous exposure–response studies have shown that inhaled grain dust and endotoxin produce similar physiologic and biologic effects in humans (12) and mice (13); the concentration of endotoxin in grain dust plays an important role in the acute biological response to grain dust in humans (12) and mice (13); a competitive antagonist for LPS (*Rhodobacter sphaeroides* diphosphoryl lipid A) reduces the inflammatory response to inhaled grain dust in mice (14); and genetic or acquired hyporesponsiveness to endotoxin substantially reduces the biological response to grain dust in mice (13). Finally, recent reports have indicated that the concentration of endotoxin in the domestic setting is related to the clinical severity of asthma (8, 15, 16).

Just as not all workers exposed to grain dust develop airway disease, not all asthmatics exposed to contaminated bioaerosols develop exacerbations of their lung disease. There is

(*Received in original form August 28, 1998 and in revised form February 2, 1999*)

Supported by grants from the Department of Veterans Affairs (Merit Review), the National Institute of Environmental Health Sciences (ES05605, ES97004, and ES07498), and the National Heart, Lung and Blood Institute (HL02950, HL59324, and HL62628).

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Am J Respir Crit Care Med Vol 160. pp 297–303, 1999
Internet address: www.atsjournals.org

considerable variation in responsiveness to inhaled LPS in the literature (9, 17). Based on these observations, we hypothesized that a range of sensitivity to the physiologic responses to inhaled endotoxin may exist. Moreover, we believe that the physiologic response to inhaled endotoxin is mediated by biological factors that recruit neutrophils to the airway. To test this hypothesis, we developed a protocol for graded exposure to inhaled LPS. We examined normal, nonatopic, nonasthmatic individuals who were lifetime nonsmokers, and studied their physiologic response to inhaled endotoxin. In addition, *in vitro* correlates of these groups were examined: alveolar macrophages and peripheral blood monocytes isolated from members of the hyporesponsive group and stimulated with LPS, and the amounts of IL-6 and IL-8 released were measured. IL-6 and IL-8 were examined because of their recognized importance in LPS-induced airway disease (10, 12). The findings of this study have implications for the variable development of airway inflammation and airflow obstruction in individuals exposed to bioaerosols contaminated with endotoxin.

METHODS

Subjects

Our study population consisted of 72 healthy adult volunteers (26 men, 46 women) age 18 to 50 yr. Exclusion criteria included any history of tobacco use, cardiac or pulmonary disease, or allergies. After written informed consent was obtained, all subjects were screened with spirometry, inhalation challenge with histamine, skin testing for common aeroallergens, chest radiograph, and electrocardiogram. All participants had normal screening studies (including provocative concentration of histamine causing a 20% reduction in FEV_1 [PC_{20}] > 32 mg/ml; there was no difference between the groups in the percent change in FEV_1 following inhalation of the maximal concentration of histamine; *see* Table 1), were on no medications (except birth control), and had no significant acute or chronic cardiopulmonary disease or occupational exposures. Our selection criteria and exposure protocol were reviewed and approved by the Human Subjects Use Committee of the University of Iowa.

Endotoxin

Solutions of endotoxin for inhalation purposes were prepared according to a standard protocol using lyophilized *Escherichia coli* (serotype 0111:B4; Sigma Chemical Co., St. Louis, MO) LPS. These solutions of LPS were resuspended in sterile Hanks' balanced salt solution (HBSS) (without calcium or magnesium) at a pH of 7.0 and filtered. All solutions used for inhalation were tested for sterility (bacteria and fungi) and endotoxin content (*Limulus* amebocyte lysate assay, QCL-1000; Whittaker Bioproducts, Walkersville, MD) prior to separation into individual aliquots. These aliquots were stored immediately after preparation at −70° C until used.

TABLE 1

DEMOGRAPHICS

	Phenotype			
	Sensitive ($n = 8$)	Intermediate ($n = 53$)	Hyporesponsive ($n = 11$)	p Value
Mean age, yr	28.4	27.7	30.7	NS
Sex				
Male	1 (12.5%)	17 (32.1%)	8 (72.7%)	0.016
Female	7 (87.5%)	36 (67.9%)	3 (27.3%)	
Weight, kg	68.5	74.0	75.6	NS
Race				
White	5	47	11	NS
African-American	1	5		
Asian	2	1		
Histamine response, L	0.20 ± 0.03	0.11 ± 0.02	0.14 ± 0.06	NS

Definition of abbreviation: NS = not significant.

Inhalation Challenge Protocol

All subjects were exposed by inhalation challenge to buffered sterile saline (HBSS) followed by increasing concentrations of LPS. The solutions were delivered via a DeVilbiss 646 nebulizer powered by compressed air at 30 psi (DeVilbiss Co., Somerset, PA) and a Rosenthal dosimeter (Laboratory for Applied Immunology, Baltimore, MD). After the HBSS, subsequent inhalations delivered increasing doses of LPS according to the following schedule: 0.5 μg, 1.0 μg, 2.0 μg, 3.0 μg, 5.0 μg, 10 μg, and 20 μg. Thus, the entire protocol delivered a total of 41.5 μg of LPS.

Physiologic Measurements

A spirometer (model S600; Spirotech, Atlanta, GA) was used to assess pulmonary function. Subjects were positioned upright in a chair and were using noseclips. Baseline spirometry was recorded after inhalation of saline, and then 1, 10, 20, and 30 min after inhalation of each dose of LPS, and compared with the postsaline baseline spirometry. If the study subject's FEV_1 was greater than 80% of the baseline measurement at the final assessment (30 min postsaline), the inhalation challenge was continued and the next dose of LPS was administered. The challenge test was terminated when any of the following criteria had been met: (*1*) the subject did not wish to continue for any reason; (*2*) the subject's FEV_1 decreased 20% or greater from baseline; or (*3*) a cumulative dose of 41.5 μg had been achieved.

Assignment of Phenotype

Study subjects were categorized as having a sensitive, intermediate, or hyporesponsive airway response to inhaled LPS based on our prior clinical experience and a review of the relevant literature. In the course of our previous investigations in grain dust–induced airway disease to inhaled LPS, we exposed a large number of study subjects to inhaled LPS (10, 12, 18–21). Our experience indicates that most healthy nonasthmatic study subjects develop airflow obstruction ($FEV_1 \leqslant 80\%$ of the preexposure value) when challenged with 40 μg of LPS, although others have found a significant change (8.3% decline) in normal subjects only after 200 μg (9). In addition, while we have found that subjects with mild intermittent asthma develop airflow obstruction (decline in $FEV_1 \geqslant 20\%$) when challenged with 15 to 20 μg of inhaled LPS, Michel and colleagues found a significant decline in FEV_1 (6.7%, range 4.5 to 11%) in asthmatics after inhalation of 20 μg of LPS (17). Differences in the type of endotoxin (*E. coli* versus *Enterobacter agglomerans*) and inhalation protocols may account for the differences noted in the magnitude of the physiologic response to inhaled LPS. However, based on our experience, we anticipated that most healthy, nonasthmatic subjects participating in the incremental LPS inhalation protocol would develop airflow obstruction ($FEV_1 \leqslant 80\%$ of preexposure value) during the course of the LPS challenge and certainly after inhaling a total of 41.5 μg of LPS. *A priori*, we decided to categorize subjects as "sensitive" if they decreased their FEV_1 by 20% or more after inhaling $\leqslant 6.5$ μg, or "hyporesponsive" if they had a $\leqslant 10\%$ decline in their FEV_1 after inhaling a total of 41.5 μg of LPS. We reasoned that these two extreme categories (sensitive and hyporesponsive) represented distinct and unusual airway responses to the proposed inhalation challenge with LPS. Subjects were classified as having an "intermediate" response to inhaled LPS if they did not satisfy the criteria for the sensitive or hyporesponsive categories. Although these definitions are somewhat arbitrary, and we may exclude a number of "sensitive" and "hyporesponsive" individuals by the stringency of these criteria, we wished to be able to define populations of extreme responders who clearly differed in their response to inhaled LPS.

Isolation of Alveolar Macrophages and Blood Monocytes

All "sensitive" (n = 8) and "hyporesponsive" (n = 11) subjects, identified on the basis of their physiologic response to LPS inhalation, were encouraged to participate in a study of the *in vitro* cellular response to LPS. Seven (88%) sensitive and five (45%) hyporesponsive

subjects consented, and underwent phlebotomy and bronchoalveolar lavage, as previously described (22). Briefly, five 25-ml aliquots of sterile, warmed saline were instilled into the lung through a wedged flexible fiberoptic bronchoscope and withdrawn under low suction. The first 25-ml lavage from each site was discarded; the lavage was performed in three subsegments in each individual. Lavage fluid was processed immediately, and total and differential cell counts carried out. In all cases, alveolar macrophages comprised > 95% of the harvested cells. Phlebotomy (180 ml) was carried out by peripheral venipuncture; mononuclear cells were isolated using a Ficoll-Hypaque density gradient and further purified by adhesion.

Cell Culture

The macrophages and monocytes were cultured at a density of 1×10^6 cells/ml in RPMI 1640 containing 0.3 mg/ml L-glutamine, and 5% endotoxin-free fetal calf serum (Hyclone Laboratories, Logan, UT). Cells were incubated in an atmosphere of 95% humidified air, 5% CO_2, at 37° C. Cells were stimulated with LPS (10 ng/ml) and harvested after 3 (for RNA isolation) or 24 h (for protein measurements in culture supernatant) in culture. Supernatants were immediately frozen at −70° C for subsequent cytokine analysis. Cell pellets were suspended in phenol and snap-frozen in liquid nitrogen for subsequent purification of RNA.

Cytokine Assessment

Culture supernatants were assayed for cytokine release by sensitive and specific sandwich ELISA using antibody pairs for IL-6 and IL-8 (R&D, Minneapolis, MN), in accordance with R&D protocols.

RNA Isolation and Ribonuclease (RNase) Protection Assay

When sufficient cells were obtained (in six sensitive and four hyporesponsive subjects), total RNA was extracted from isolated human macrophages and monocytes using the single-step method (23), lysing cells in RNA STAT-60 (Tel-Test B, Friendswood, TX). The composition of RNA STAT-60 includes phenol and guanidinium isothiocyanate in a monophase solution. Cells in RNA STAT were homogenized, chloroform was added, and total RNA was precipitated from the aqueous phase by addition of isopropanol. The RNA pellet was washed with ethanol and solubilized in RNase-free water. Measuring the ratio and absorbencies at 260 and 280 nm quantitated the yield and purity of the RNA. Gene transcripts were detected using a multiprobe RNase protection assay (RiboQuant, Multi-Probe RNase Protection Assay System; Pharmingen, San Diego, CA) as previously described (24). Custom probe sets that included DNA templates for cytokines (IL-1α, IL-1β, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p35, IL-12p40, interferon gamma [IFN-γ], transforming growth factor-β1 [TGF-β1], and tumor necrosis factor-α [TNF-α]) and housekeeping genes (glyceraldehyde-3-phosphate dehydrogenase [GAPDH] and L32) were used to generate antisense cRNA transcripts. Ten micrograms of total RNA was hybridized with a ^{32}P-labeled antisense cRNA probe set in a solution hybridization buffer for 14 h at 56° C. The nonhybridized single-stranded RNA was digested with a mixture of RNase A and T1. The remaining protected RNA fragments were extracted with phenol:chloroform: isoamyl alcohol (25:24:1) and ethanol precipitated. The protected hybridization products were separated on a 5% acrylamide/8 M urea gel. The gel was dried on a vacuum gel dryer at 80° C, wrapped in plastic wrap and exposed to X-ray film overnight at −70° C to visualize the protected hybridized probe.

Statistical Analysis

After categorization into "sensitive," "intermediate," or "hyporesponsive" groups, two-way analysis of variance (ANOVA) with *post hoc* Tukey testing and two-sample *t* tests were used to compare the three groups. Least-squares linear regression was used to calculate the dose–response slope of each group, and both evaluation of intraclass correlation coefficient and the reliability testing method of Bland and Altman were used to assess the reproducibility of repeated measures of response to inhaled endotoxin (25). Fisher exact two-tailed test was used to test for a general sex association with sensitive or hyporesponsive phenotype. Nonparametric Mann-Whitney tests were employed in noting differences in percent baseline FEV_1 between males and females at various levels. Finally, two sample Mann-Whitney U tests with 95% confidence intervals (CI) were calculated for the mean IL-6 and IL-8 concentrations obtained by the biologic assay described previously. The original population, but not the subpopulations, was found to encompass a normal distribution on testing.

RESULTS

Frequency Distribution of LPS PD_{20}

Marked differences in the response to inhaled LPS were observed among our study subjects. Although 40 individuals had at least a 20% decline in their FEV_1, eight "sensitive" subjects had a 20% or greater decline in their FEV_1 after inhaling a total of 6.5 μg or less of LPS, whereas 11 "hyporesponsive" subjects maintained an FEV_1 of at least 90% of their baseline value even after inhaling 41.5 μg of LPS (Figure 1). Of note, 21 subjects had a 10 to 20% reduction in their FEV_1 after inhaling a total of 41.5 μg, and were all classified as intermediate responders.

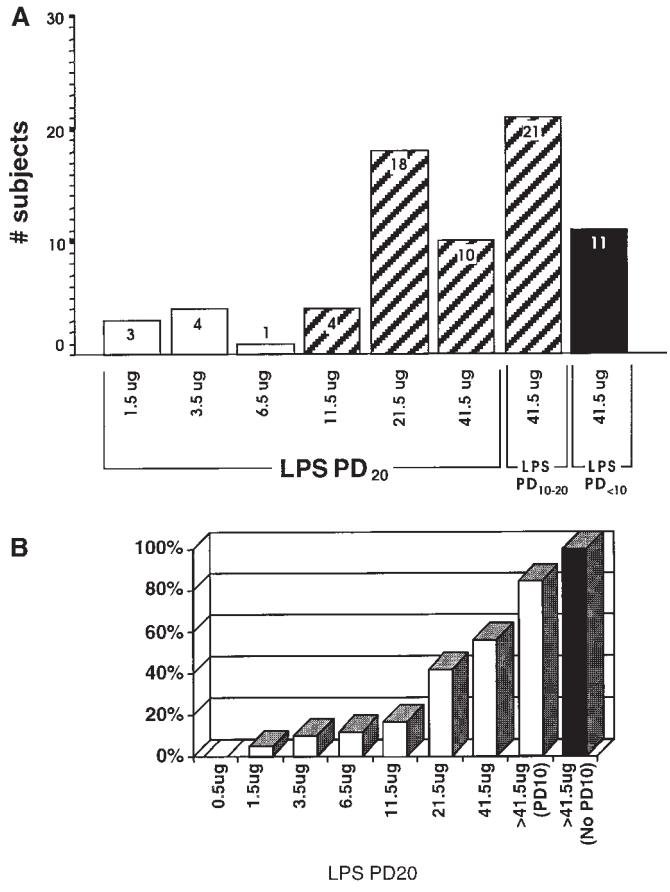

Figure 1. Frequency distribution and cumulative histograms of dose of inhaled LPS that causes a fall of ⩾ 20% from the baseline FEV_1 (PD_{20}). Seventy-two healthy subjects received a graded inhalation challenge with increasing doses of inhaled LPS. Each dose of LPS was followed by spirometry measurements (1, 10, 20, and 30 min) before administration of the subsequent dose. Eight "sensitive" subjects (*open bars*) had a $PD_{20} \leqslant 6.5$, and 11 "hyporesponsive" subjects (*solid bars*) maintained an FEV_1 of at least 90% of their baseline after inhaling 41.5 μg of LPS. (*A*) Histogram demonstrating numbers of subjects by PD_{20}. (*B*) Cumulative percentage of subjects reaching PD_{20} after various exposures to LPS.

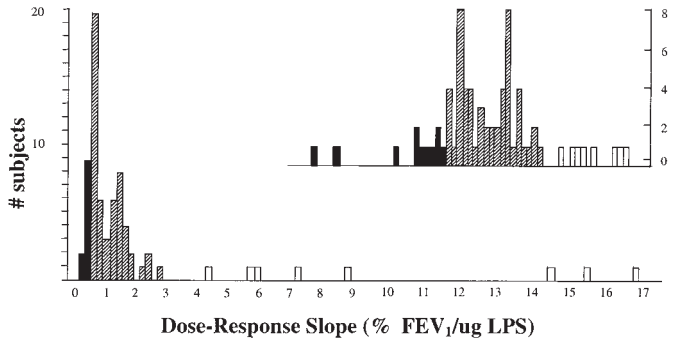

Figure 2. Frequency distribution histogram of dose–response slope (%$\Delta FEV_1/\mu g$ LPS). The %$\Delta FEV_1/\mu g$ LPS was calculated after administration of the cumulative LPS dose that either resulted in at least a 20% decline in FEV_1, or after a cumulative dose of 41.5 μg. Eleven hyporesponsive (*solid bars*) and eight sensitive (*open bars*) individuals were defined on the basis of their response to inhaled LPS. The remaining 53 individuals (*striped bars*) were classified as intermediate sensitivity. Dose–response histogram. (*Inset*) Log transformation of data demonstrating a normal, unimodal distribution.

Dose–Response to Inhaled LPS

We next derived the dose–response slope for each subject, which was calculated by the expression: percent decline FEV_1/dose, where percent decline FEV_1 was defined as the total percentage decline in FEV_1 (from the baseline value) after the final LPS dose administered, and the dose was defined as the cumulative LPS dose (Figure 2). Each subject was classified as one of three phenotypes (sensitive, intermediate, or hyporesponsive), as described in METHODS. Eight sensitive subjects had a dose response of 4.5 to 17% drop in $FEV_1/\mu g$ LPS inhaled, and 11 hyporesponsive subjects had a dose response of 0 to 0.2% drop in $FEV_1/\mu g$ LPS inhaled. These classifications corresponded to the lower fifteenth and the upper tenth percentiles, respectively. Log transformation of the dose–response data demonstrates a normal unimodal distribution (Figure 2B). The dose–response slopes of the sensitive and hyporesponsive groups are well separated from those of the intermediate group (Figure 3).

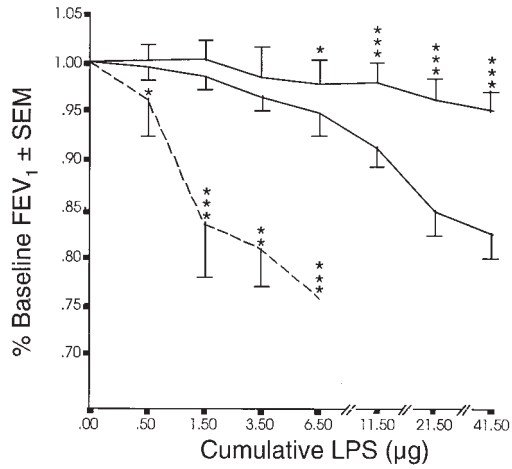

Figure 3. Dose–response curves of the sensitive (*dashed line*), intermediate (*dotted line*), and hyporesponsive (*solid line*) groups to inhaled LPS are displayed with 95% CI. Two-way ANOVA demonstrates that the lines are overall significantly different. *Post hoc* testing reveals that the sensitive group is significantly different from the intermediate group at doses of $\geq$ 0.5 μg LPS. The hyporesponsive group is significantly different from the intermediate group at doses of $\geq$ 6.5 μg LPS. *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$ versus intermediate group.

Demographics of Participants

Of the 126 individuals who responded to the request for volunteers, 98 were screened, and 76 subjects met the inclusionary criteria and thus qualified for the study. Based on the above criteria, eight subjects were classified as sensitive, 53 as intermediate, and 11 as hyporesponsive (Table 1). Four subjects elected to withdraw from the study after the completion of screening for reasons other than the defined endpoints, and were not included in subsequent analyses. A significant sex difference was noted in the groups; seven of eight sensitive subjects were female, whereas eight of 11 hyporesponsive subjects were male ($p = 0.016$). Among all study subjects, the dose–response curves of males and females were significantly different at 6.5, 11.5, and 41.5 μg of LPS, cumulative dose (Figure 4). There were no differences between the three study groups in age, weight, or race.

Reproducibility of Dose–Response in Individuals

In order to determine if the LPS response phenotype (sensitive, intermediate, or hyporesponsive) of an individual was reliable, we repeated the LPS inhalation protocol on 17 individuals: five sensitive, four intermediate, and eight hyporesponsive subjects. These repeat exposures were all performed at least 4 wk after the initial inhalation challenge. Overall reliability of the continuous variable, percent baseline FEV_1/dose appeared strong with a statistically significant intraclass correlation coefficient of nearly 0.60. This was confirmed with the reliability testing method of Bland and Altman (25). On an individual basis, all hyporesponsive subjects and four of five sensitive subjects had nearly identical curves and maintained their original classification (Figure 5). Although three subjects (numbers 9, 45, and 56) originally classified as intermediate responders were classified as being sensitive on repeated challenge, these repeat studies showed that the phenotypes are very reliable at the extremes, and that this method of phenotyping is reproducible and reliable.

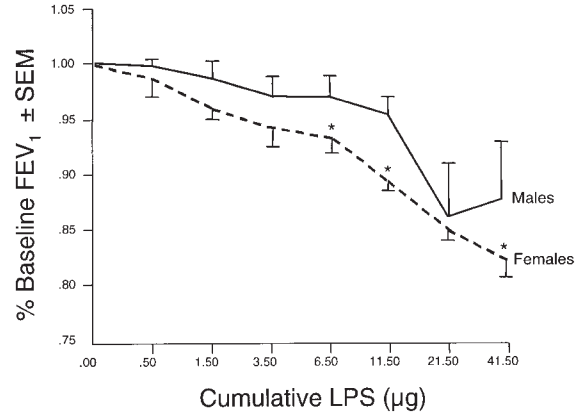

Figure 4. Dose–response curves of male and female subjects: dose inhaled LPS versus FEV_1 relative to baseline FEV_1. Dose–response curves (mean ± SEM) of the female (*dashed line*) and male (*solid line*) subjects to inhaled LPS are displayed. The female group is significantly different from the male group at 6.5, 11.5, and 41.5 μg of LPS. *$p < 0.05$, male versus female groups.

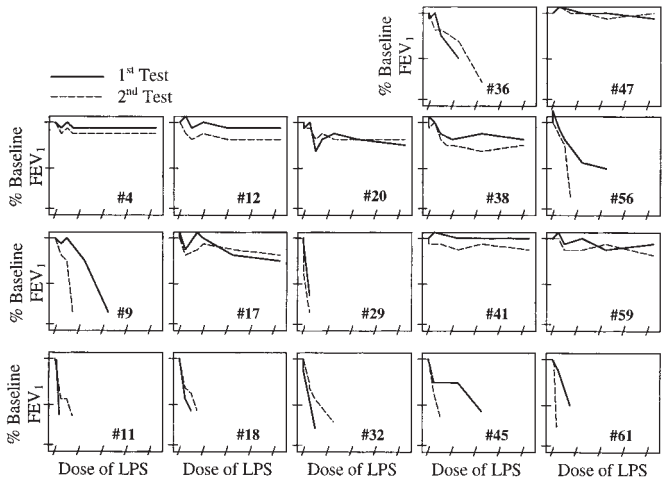

Figure 5. Reliability of endotoxin dose–response curves. Five sensitive, four intermediate, and eight hyporesponsive subjects underwent repeat graded LPS inhalation challenges to assess the reliability of the dose–response relationship between inhaled LPS and change in FEV_1. Analysis of reliability (25) demonstrates that the repeated test is reliable. This is confirmed by the intraclass correlation coefficient. When stratified by category all eight hyporesponsive subjects and four of five sensitive subjects maintained their original classification. Three intermediate subjects (numbers 9, 45, and 56) were classified as sensitive by repeat testing.

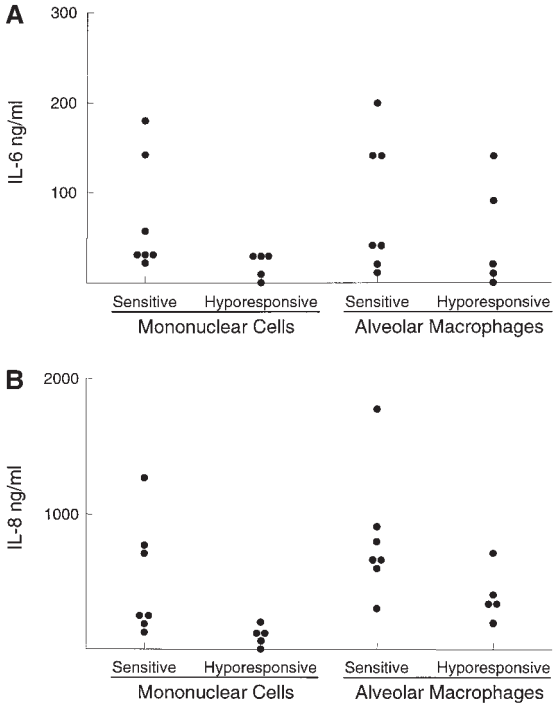

Figure 6. Scatter plot of LPS-stimulated release of IL-6 (*A*) and IL-8 (*B*). Alveolar macrophages and peripheral blood monocytes from sensitive (n = 7) and hyporesponsive (n = 5) individuals were cultured for 24 h in complete medium with LPS (10 ng/ml). (*A*) The amount of IL-6 released from stimulated monocytes is significantly greater in cells from sensitive than in those from hyporesponsive individuals ($p < 0.05$). (*B*) The amount of IL-8 released from stimulated monocytes as well as from macrophages was significantly greater in cells from sensitive than in those from hyporesponsive individuals ($p < 0.05$).

Biologic Correlates of Sensitive and Hyporesponsive Phenotypes

We next investigated whether the sensitive and hyporesponsive phenotypes, defined by the development of airway obstruction after inhalation of LPS, correlated with abnormal responses to LPS by inflammatory cells. For these studies, we obtained alveolar macrophages, by bronchoalveolar lavage, and peripheral blood monocytes from seven sensitive and five hyporesponsive subjects. Cells were cultured in the presence of LPS (10 ng/ml), and harvested 24 h later. The culture supernatants were studied for cytokine release, and the cells were harvested for evaluation of messenger RNA (mRNA) content. We found that both macrophages and monocytes from hyporesponsive individuals demonstrated an attenuated release of IL-6 and IL-8, in comparison with cells from sensitive subjects (Table 2 and Figure 6). RNase protection assay of RNA isolated from alveolar macrophages or peripheral monocytes (from six sensitive and four hyporesponsive subjects), however, demonstrated no difference between the two groups for these cytokines or others (Figure 7).

TABLE 2

CYTOKINE RELEASE

		Phenotype	
	Cytokine	Sensitive ($n = 7$) Mean ± SEM Median (Range)	Hyporesponsive ($n = 5$) Mean ± SEM Median (Range)
Alveolar macrophages	IL-6 (ng/ml)	87.9 ± 28.2 46 (12–201)	52.8 ± 26.9 18 (3–140)
	IL-8 (ng/ml)	770.7 ± 133.9 671 (305–1,454)	414.6 ± 86.7* 365 (204–732)
Peripheral blood monocytes	IL-6 (ng/ml)	71.7 ± 24.0 32 (27–181)	19.0 ± 6.2* 25 (0–32)
	IL-8 (ng/ml)	509.1 ± 177.5 274 (125–1,459)	107.2 ± 32.7* 123 (0–146)

*$p < 0.05$, sensitive versus hyporesponsive.

DISCUSSION

These results indicate that most healthy, nonatopic, nonasthmatic, volunteer subjects develop significant airflow obstruction when challenged with up to 40 μg of inhaled LPS. Importantly, our results also show that the incremental LPS inhalation challenge can reliably identify a small percentage of individuals who are either exquisitely sensitive or hyporesponsive to inhaled LPS. Our results also indicate that the physiologic response to inhaled LPS appears to be significantly influenced by sex; women are significantly more likely to be in the sensitive group and men in the hyporesponsive group. These LPS phenotypes (sensitive, intermediate, and hyporesponsive) are reproducible, because repeated testing of individuals demonstrates no significant shift in the dose–response to inhaled LPS. Finally, we have demonstrated that a difference exists in the *in vitro* response to LPS by inflammatory cells among members of the sensitive and hyporesponsive groups. These findings suggest that individuals have a unique physiologic response to inhaled endotoxin that appears to correlate with an *in vitro* ability to respond to LPS. This LPS phenotype may be influenced by genetic factors, sex differences, other exposures, or comorbid conditions.

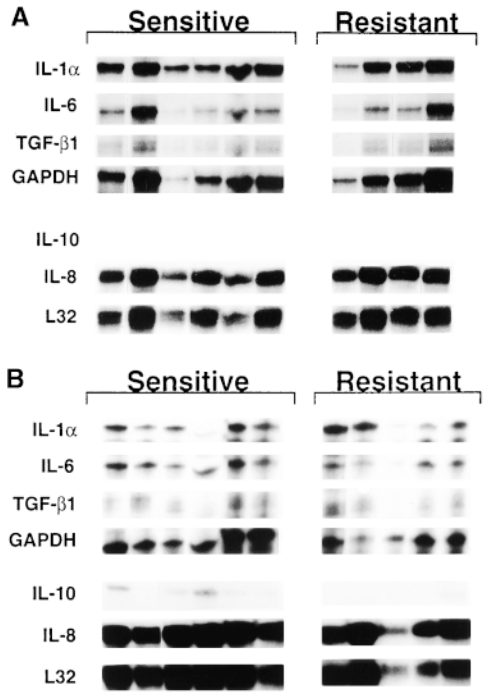

Figure 7. RNase protection assays of total RNA obtained from alveolar macrophages and peripheral blood monocytes from sensitive and hyporesponsive individuals, stimulated *in vitro* with LPS. Sufficient alveolar macrophages (*A*) and peripheral blood monocytes (*B*) to isolate RNA and perform RNase protection assays (RPAs) were obtained from hyporesponsive (n = 4) and sensitive (n = 6) individuals. The cells were stimulated *in vitro* with LPS (10 ng/ml), and harvested 3 h later. RNA was isolated from the cells and RPAs were carried out. Probes for IL-1α, IL-6, TGF-β1, IL-10, and IL-8 showed no difference in transcription levels of these cytokines between the two groups. Equivalent amounts of RNA were examined in each sample, as judged by the signal for GAPDH (a housekeeping protein) or L32 (a ribosomal subunit protein) in the probe sets.

Differences in genetic susceptibility may account for our study findings. In mice, genetic differences in susceptibility to the physiologic response to LPS are well established. The murine *Lps* response gene has been shown to be a single gene with classic Mendelian genetics. This gene has been mapped to mouse chromosome 4 (26–28), and recent evidence suggests that the LPS response gene in mice is Tlr-4 (29). Tlr-4 is a member of the Toll family of genes, all of which appear to be important in innate immunity. For instance, Tlr-2 has recently been shown to enhance the sensitivity of cells to LPS (30) and Tlr-4 has sequence homology to the IL-1 receptor (29). Thus, polymorphisms or mutations in these genes may alter the biologic and physiologic response to endotoxin. Humans clearly demonstrate a broad spectrum in the clinical response to inhaled endotoxin (31). Our current study suggests that this spectrum of sensitivity is both widespread and reproducible, and lends credence to the likelihood that specific genetic changes may enhance or suppress the inflammatory response to LPS.

Another factor that we noted as associated with the response to LPS inhalation was sex; women were more likely than men to develop airflow obstruction after the inhalation of lower doses of LPS. There are a number of acquired and genetically determined differences between the sexes. For example, it is well established that women have lower cardiovascular mortality than men do, at least in part because of differences in serum cholesterol levels; women have higher levels of high-density lipoprotein (HDL) cholesterol and lower levels of low-density lipoproteins (LDL) cholesterol than do men, especially in the premenopausal age. LDL can bind LPS and, *in vitro*, diminishes LPS-stimulated cellular inflammation (32). Although LDL has not been reported in the airspaces of the lung, the composition of lipids in the circulation may alter the composition of lipid binding proteins in the lung, which may have a profound effect on the biological activity of inhaled LPS. This area clearly needs further investigation.

Comorbid diseases, such as asthma, may also account for differential susceptibility to inhaled LPS. Although we excluded asthmatics from our study, asthma severity has been linked to exposure to pollutants, such as particulate matter, ozone, and endotoxin, as well as allergen contact. Mediators released in the airways after endotoxin exposure that may account for the development of airway inflammation include IL-1β, TNF-α, IL-6, and IL-8 from macrophages, recruited neutrophils, and constitutive cells of the lung. Inhalation of endotoxin may influence the airway response to other bronchospastic agents. For instance, animal studies have been carried out showing that inhalation of endotoxin by rats (33) causes airway hyperreactivity to inhaled methacholine. The inflammatory responses induced by endotoxin and by allergens have also been examined in animal studies. Macari and colleagues noted that prior administration of endotoxin to sensitized guinea pigs causes increased eosinophilic inflammation after allergen challenge (34) suggesting specific interactions between the inflammatory responses induced by both stimuli. Asthmatic individuals develop airflow obstruction at lower concentrations of inhaled endotoxin (17) and inhalation of allergens increases the lung's biological responsiveness to endotoxin (35). Interestingly, inhaled allergens appear to increase the concentration of lipopolysaccharide binding protein (LBP), which allows the lung inflammatory cells to respond to very low concentrations of endotoxin that are commonly present in the airways of uninfected lungs (35). This may explain why asthmatic patients exposed to endotoxin by inhalation develop a pronounced inflammatory response characterized by increased release of TNF-α and airway neutrophilia (8). As endotoxin content of dust in the home does not correlate with allergen content (15) exposure to inhaled endotoxin may account for some flares of atopic asthma that occur without change in allergen exposure.

This present study is significant in that we have, for the first time, identified distinct phenotypes of endotoxin responsiveness in nonatopic, nonasthmatic individuals. These phenotypes appear to be significantly influenced by sex, and females were more sensitive than males to the physiologic effects of inhaled endotoxin. In addition, there were *in vitro* differences in responsiveness to LPS between the monocytes and alveolar macrophages from sensitive and hyporesponsive groups. These findings suggest that genetic factors, sex differences, other exposures, or comorbid conditions may play a role in the biologic responses to LPS. Further clarification of the sensitive and resistant phenotypes will help to identify the etiologic factors responsible for these physiologic differences.

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